



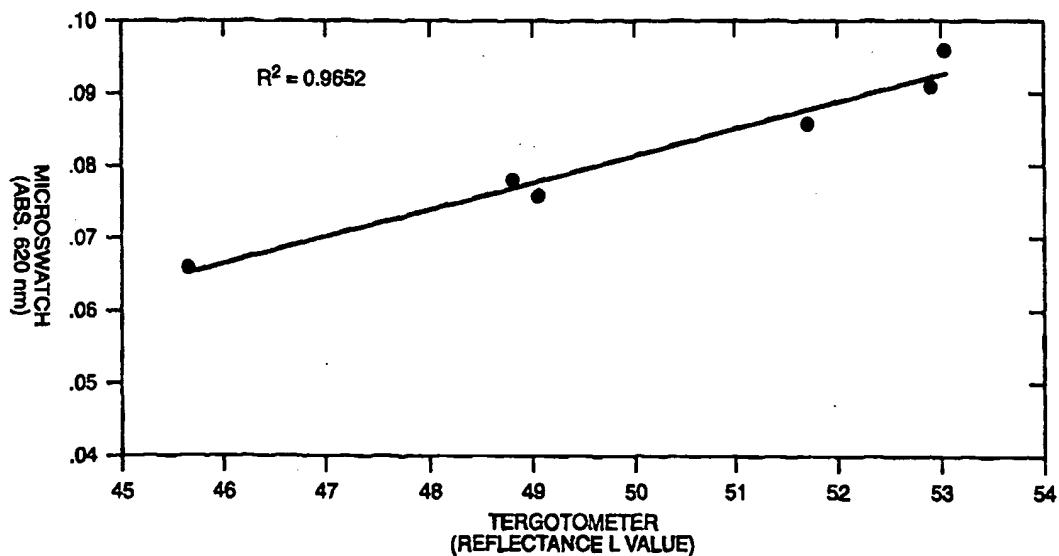
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/00	A2	(11) International Publication Number: WO 99/34011 (43) International Publication Date: 8 July 1999 (08.07.99)
<p>(21) International Application Number: PCT/US98/27629</p> <p>(22) International Filing Date: 23 December 1998 (23.12.98)</p> <p>(30) Priority Data: 60/068,796 24 December 1997 (24.12.97) US</p> <p>(71) Applicant (<i>for all designated States except US</i>): GENENCOR INTERNATIONAL, INC. [US/US]; 4 Cambridge Place, 1870 South Winton Road, Rochester, NY 14618 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): SCHELLENBERGER, Volker [DE/US]; 1747 Sequoia Avenue, Burlingame, CA 94010 (US). NAKI, Donald, P. [US/US]; 4815 – 25th Street, San Francisco, CA 94118 (US). COLLIER, Katherine, D. [US/US]; 915 Wilmington Way, Redwood City, CA 94062 (US). KELLIS, James, T., Jr. [US/US]; 111 Tan Oak Drive, Portola Valley, CA 94028 (US). NADHERNY, Joanne [US/US]; 681 Arguello No. 6, San Francisco, CA 94118 (US).</p> <p>(74) Agent: ANDERSON, Kirsten, A.; Genencor International, Inc., 925 Page Mill Road, Palo Alto, CA 94304–1013 (US).</p>		(81) Designated States: AL, AM, AT, AT (Utility model), AU (Petty patent), AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: AN IMPROVED METHOD OF ASSAYING FOR A PREFERRED ENZYME AND/OR PREFERRED DETERGENT COMPOSITION



(57) Abstract

An improved method for assaying the wash performance of new enzymes and/or new detergent formulations is described.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

**AN IMPROVED METHOD OF ASSAYING FOR A PREFERRED ENZYME AND/OR
PREFERRED DETERGENT COMPOSITION**

Background of the Invention

5 Enzymes are a necessary part of many of the detergent compositions that are currently on the market and the inclusion of enzymes in detergent compositions will undoubtedly increase in the future. One of the most important challenges facing a detergent manufacturer today is the identification of new and improved enzymes and detergent compositions. New enzymes can and commonly do include variants of known 10 enzymes.

Several factors can affect the determination of the "improvement" of a new enzyme over an precursor enzyme, i.e., the enzyme itself, the wash conditions, and the detergent composition that the enzyme is to be mixed with. For example, an enzyme that performs well in one detergent composition may not perform as well in another. Similarly, an 15 enzyme and/or detergent composition may perform well under one set of wash conditions, i.e., Japanese, but not another, i.e., North American. However, identifying a new and improved enzyme or detergent composition can be a time consuming task. For example, in the wake of improved technology that can allow a researcher to produce large numbers of variants in a very short time, it has become critical for the researcher to be able to assay 20 those variants rapidly, efficiently and effectively.

Summary of the Invention

The present invention provides a method of assaying for a preferred enzyme including providing a swatch that includes a piece of material and a stain. The stain is then 25 fixed to the material and a smaller swatch can be removed from the swatch. Alternatively, the smaller swatch can be removed from the larger swatch and then the stain can be fixed. Next, an enzyme is applied to the swatch or smaller swatch and they are incubated together.

The method can further include measuring the degree of removal of the stain from 30 the material. The method can also include agitating the smaller swatch and enzyme during incubation. The material can be, for example, cotton, polyester or mixtures of natural and synthetic fibers. The stain can include blood, milk, ink, grass, gravy, chocolate, egg, cheese, clay, pigment, oil, and combinations thereof. The enzyme can be applied to the swatch or smaller swatch in combination with a detergent ingredient.

35 The present invention also provides a method of assaying for a preferred detergent composition including providing a swatch that includes a piece of material and a stain. The

stain is then fixed to the material and a smaller swatch can be removed from the swatch. Alternatively, the smaller swatch can be removed from the larger swatch and then the stain can be fixed. Next, a detergent composition is applied to the swatch or smaller swatch and they are incubated together.

5 The method can further include measuring the degree of removal of the stain from the material. The method can also include agitating the swatch or smaller swatch and detergent composition during incubation. The material can be, for example, cotton, polyester or mixtures of natural and synthetic fibers, cellulose and derivatives of cellulose. The stain can include blood, milk, ink, grass, spinach, wine, tea, gravy, chocolate egg, 10 cheese, clay, pigment, oil, and combinations thereof. The detergent composition can be applied to the swatch or smaller swatch in combination with an enzyme.

Brief Description of the Drawing

Figure 1 shows the correlation between the results of testing six protease variants 15 in a tergotometer test according to the method of the present invention.

Detailed Description of the Invention

One aspect of the present invention is directed to a method of assaying for a preferred enzyme that includes providing a swatch of material - a piece of material and a 20 stain - then fixing the stain to the material, optionally removing a smaller swatch from the swatch, applying the enzyme to the swatch or smaller swatch and incubating them.

A further aspect of the invention is directed to a method of assaying for a preferred detergent composition that includes providing a swatch of material that includes a piece of material and a stain, then fixing the stain to the material, optionally removing a smaller 25 swatch from the swatch, applying the detergent composition to the swatch or smaller swatch and incubating them.

Another aspect of the invention is directed to a method of assaying the release of a stain from a blood/milk/ink (BMI)-stained swatch including measuring the absorbance or fluorescence of, for example, the ink, labeled blood or labeled milk in the supernatant after 30 the swatch has been incubated with an enzyme or detergent composition.

In addition, an aspect of the invention includes a method of agitating the microtiter plate to a sufficient degree to assure complete and efficient incubation of the enzyme with the smaller swatch. The method includes applying a plate sealer to the top of the microtiter plate and then clamping another lid on top of the plate sealer.

35 Any enzyme or combination of enzymes may be used in the present invention. Preferred enzymes include those enzymes capable of hydrolyzing substrates, e.g. stains.

These enzymes are known as hydrolases which include, but are not limited to, proteases (bacterial, fungal, acid, neutral or alkaline), amylases (alpha or beta), lipases, cellulases and mixtures thereof. Particularly preferred enzymes are subtilisins and cellulases. Most preferred are subtilisins such as described in U.S. Patent 4,760,025, EP Patent 130 756

5 B1 and EP Patent Application WO 91/06637, which are incorporated herein by reference, and cellulases such as Multifect L250™ and Puradax™, commercially available from Genencor International. Other enzymes that can be used in the present invention include oxidases such as laccases, transferases, dehydratases, reductases, hemicellulases and isomerases.

10 A "swatch" is a piece of material such as a fabric that has a stain applied thereto. The material can be, for example, fabrics made of cotton, polyester or mixtures of natural and synthetic fibers. The swatch can further be paper such as filter paper or nitrocellulose or a piece of a hard material such as ceramic or glass. The stain can be blood, milk, ink, grass, tea, wine, spinach, gravy, chocolate egg, cheese, clay, pigment, oil, or mixtures of 15 these compounds.

A "smaller swatch" is a piece of the swatch that has been cut or otherwise removed from the swatch of material either before or after fixing the stain to the swatch and can, for example, fit into the well of a 24, 48 or 96 well microtiter plate. The "smaller swatch" can also be made by applying a stain to a small piece of material. Preferably, the smaller 20 swatch is a piece of fabric with a stain 5/8" in diameter, more preferably, the smaller swatch is 0.25" in diameter.

When, for example, untreated BMI swatches are washed in detergent without bleach, a large portion of the ink is released even without the help of a protease. Adding a protease leads to a small increase in ink release which can be hard to quantify over the 25 large background. The present invention provides a treatment protocol which allows one to control the degree of fixation of a stain. As a result, it is possible to produce swatches which, for example, release varying amounts of ink when washed in the absence of protease. The use of fixed swatches leads to a dramatic improvement of the signal-to-noise ratio in the wash assays. Furthermore, by varying the degree of fixation one can 30 generate stains which give optimum results under the various cleaning conditions.

Swatches having stains of known "strength" on various types of material are commercially available (EMPA, St. Gallen, Switzerland; wfk - Testgewebe GmbH, Krefeld Germany; or Center for Test Materials, Vlaardingen, The Netherlands) and/or can be made by the practitioner (Morris and Prato, Textile Research Journal 52(4):280-286 (1982)).

35 Preferred swatches are a blood/milk/ink (BMI) stain on a cotton-containing fabric, a spinach

stain on a cotton-containing fabric, or grass on a cotton-containing fabric, and chocolate/milk/soot on a cotton-containing fabric.

A stain can be fixed to a material in a number of ways. For example, the swatch can be incubated with a cross-linking agent to fix the stain. The degree of fixing can be affected by, for example, increasing or decreasing the incubation time, varying the temperature at which the incubation takes place, and/or varying the concentration of the chemical. Suitable cross-linking agents for use in the present invention include hydrogen peroxide, bleaching agents, glutaraldehyde, and carbodiimides.

In a preferred embodiment of the invention, a BMI stain can be fixed to cotton with 0.0003 - 0.3% hydrogen peroxide. Other combinations include grass or spinach fixed with 0.001-1% glutaraldehyde, gelatin and Coomassie stain fixed with 0.001-1% glutaraldehyde, or chocolate, milk and soot fixed with 0.001-1% glutaraldehyde.

An important aspect of the present invention is that the swatch and enzyme and/or detergent formulation must be well agitated during incubation. We have observed that the wash performance data is dependent on the orientation of the swatches in the wells (horizontal versus vertical), particularly in the 96-well plate. This would indicate that mixing was insufficient during the incubation period. Although there are a number of ways to ensure sufficient agitation during incubation, a plate holder in which the microtiter plate is sandwiched between two plates of aluminum can be constructed. This can be as simple as placing, for example, an adhesive plate sealer over the wells then clamping the two aluminum plates to the 96-well plate with any type of appropriate, commercially available clamps. It can then be mounted in a commercial incubator shaker. Setting the shaker to about 400 rpm results in very efficient mixing while leakage or cross-contamination is efficiently prevented by the holder.

Trinitrobenzenesulfonic acid (TNBS) can be used to quantify the concentration of amino groups in the wash liquor. This can serve as a measure of the amount of protein that was removed from the swatch (see Cayot and Tainturier, *Anal. Biochem.* 249:184-0200 (1997)). However, if a detergent or an enzyme sample leads to the formation of unusually small peptide fragments (for example, from the presence of peptidases in the sample) then one will obtain a larger TNBS signal, i.e., more "noise".

The present invention provides another and better way to measure wash performance of blood/milk/ink that is based on ink release. Proteolysis of protein on the swatches leads to the release of ink particles which can be quantified by measuring the absorbance of the wash liquor. The absorbance can be measured at any wavelength between 350 and 800 nm. In a preferred embodiment, the wavelength is measured at 410 nm. or 620 nm. The wash liquor can also be examined to determine the wash performance

on stains containing grass, spinach, gelatin or Coomassie stain. Preferred wavelengths for these stains include and 670nm for spinach or grass and 620nm for gelatin or Coomassie. For example, an aliquot of the wash liquor (typically 100 - 150ul from a 96-well microplate, for example) is removed and placed in a cuvette or multiwell microplate. This is
5 then placed in a spectrophotometer and the absorbance is read at an appropriate wavelength

The performance of samples of variant proteases (produced, for example, according to the disclosure of U.S. Patent Application Ser. No. 322,678) by the method of the present invention using TNBS and ink release detection can be compared. Several of
10 these samples show inflated wash performance when TNBS detection is used (probably due to peptidase contamination) whereas all samples result in indistinguishable signals when the absorbance of the wash liquor was measured.

The present invention can also be used to determine a preferred enzyme and/or detergent composition for dish washing, for example, using a blood/milk/ink stain on a
15 suitable substrate such as cloth, plastic or ceramic.

In a preferred embodiment of the invention, a BMI stain is fixed to cotton by applying 0.3% hydrogen peroxide to the BMI/cotton swatch for 30 minutes at 25°C or by applying 0.03% hydrogen peroxide to the BMI/cotton swatch for 30 minutes at 60°C. Smaller swatches of approximately 0.25" are cut from the BMI/cotton swatch and placed in
20 the wells of a 96 well microtiter plate. Into each well, a known mixture of a detergent composition and an enzyme such as a variant protein is placed. After placing an adhesive plate sealer onto the top of the microtiter plate, the microtiter plate is clamped to an aluminum plate and agitated for 10-300 minutes. At the end of this time, the supernatants are transferred to wells in a new microtiter plate and the absorbance of the ink at 620nm is
25 measured.

In a further preferred embodiment of the invention, a spinach or grass stain is fixed to cotton by applying 0.01% glutaraldehyde to the spinach/cotton swatch or grass/cotton swatch for 30 minutes at 25°C. Smaller swatches of approximately 0.25" are cut from the swatch and placed in the wells of a 96 well microtiter plate. Into each well, a known
30 mixture of a detergent composition and an enzyme such as a variant protein is placed. After placing an adhesive plate sealer onto the top of the microtiter plate, the microtiter plate is clamped to an aluminum plate and agitated for 10-300 minutes. At the end of this time, the supernatants are transferred to wells in a new microtiter plate and the absorbance of the ink at 670nm is measured.

35 In another preferred embodiment of the invention, a chocolate/milk/soot stain is fixed to cotton by applying 0.01% glutaraldehyde to the chocolate/milk/soot/cotton swatch

30 minutes at 25°C. Smaller swatches of approximately 0.25" are cut from the swatch and placed in the wells of a 96 well microtiter plate. Into each well, a known mixture of a detergent composition and an enzyme such as a variant protein is placed. After placing an adhesive plate sealer onto the top of the microtiter plate, the microtiter plate is clamped to an aluminum plate and agitated for 10-300 minutes. At the end of this time, the supernatants are transferred to wells in a new microtiter plate and the absorbance of the ink at an appropriate wavelength is measured.

Examples

10

Example I

A. Description of Tergotometer Protocol

15

A Tergotometer instrument manufactured by United States Testing Company was used. The machine consists of four or six 1.5 liter beakers and agitator spindles which are inserted into the beakers and rotated in a back-and forth manner at a controlled speed, typically 100 RPM, to mimic the type of agitation that occurs in commercial washing machines. The beakers are immersed in a temperature controlled water bath.

20

Each beaker was filled with one liter of deionized water to which a controlled amount of calcium and magnesium were added to mimic water hardness conditions found in the geography under study. Water hardness for North American conditions was set to 3 - 6 grains per gallon. The water bath was set to 20°C and the temperature of the water in the beakers was allowed to reach equilibrium at the testing temperature.

25

1 gram of Tide laundry detergent lacking bleach and enzyme (Procter & Gamble, Cincinnati, Ohio) was added to each beaker and allowed to mix for 1 minute while the spindles were rotating at 100 RPM. The enzyme was added to a final concentration of 0.1 micrograms per milliliter and allowed to mix for 1 minute. Blood-Milk-Ink soiled swatches 3" x 4 1/2" obtained from EMPA and modified by exposure to 3.0 % hydrogen peroxide for 30 minutes at 20°C and dried, were used. Six soiled swatches were added to each beaker and allowed to incubate for 20 minutes. After the incubation period the swatches were promptly removed from the beakers and rinsed thoroughly with water. The swatches were then placed flat on a clean lab bench to dry. When the swatches were dry, the reflectance of each swatch was measured at 3 different spots on each swatch, using a reflectance spectrophotometer with a small (typically 1/4") diameter aperture, capable of reporting results in the standard LAB scale. For BMI, it is sufficient to report only the L value, which correlates with the darkness of the stain. The L values obtained from the swatches in each pot were averaged to obtain the final reported result.

B. Description of 24-well Assay Protocol:

Blood-Milk-Ink swatches were obtained from EMPA and were exposed to 0.03 % hydrogen peroxide for 30 minutes at 60°C, then dried. Circles of ¼" diameter were cut from the dried swatches and placed one per well in a 24 well microplate. 1 gram per liter Tide laundry detergent without bleach and enzyme was prepared in deionized water, and a concentrated stock of calcium and magnesium was added to result in a final water hardness value of 6 grains per gallon. The detergent was allowed to mix for 15 minutes and was then filtered through a 0.2 micron cellulose acetate filter. Enzyme was added to the filtered detergent from a concentrated stock solution to result in a final concentration 1.25 micrograms per milliliter. The enzyme/detergent solution was then added to the appropriate wells of the microplate. The microplate was then sealed to prevent leakage and placed in a holder on an incubated shaker set to 20°C and 400 RPM and allowed to shake for one hour. The plate was then removed from the incubator/shaker and an aliquot of 20 microliters was removed from each well, and the absorbance at 620 nm wavelength was read for each aliquot and reported.

C. Six protease variants were tested according to A and B above. The results are shown in Table 1. The correlation of the data is plotted in Figure 1. The R² value is 0.9652.

Table 1
Tergotometer Microswitch

	L Value	Absorbance 620nm
A	45.62	0.066
B	48.815	0.078
C	51.755	0.086
D	49.06	0.076
E	52.915	0.091
F	53.065	0.096

Claims

1. A method of assaying for a preferred enzyme comprising:
 - a) providing a swatch of material comprising a piece of material and a stain;
 - b) fixing the stain to the material;
 - c) applying an enzyme to the swatch; and
 - d) incubating the swatch and enzyme.
2. The method of claim 1, further comprising measuring the degree of removal of the stain from the material.
3. The method of claim 1, wherein the enzyme is selected from the group consisting of a protease, a cellulase, an amylase, a laccase, and a lipase.
4. The method of claim 1, wherein the material is selected from the group consisting of a fabric, plastic, glass or ceramic.
5. The method of claim 1, wherein the stain is selected from the group consisting of blood, milk, ink, grass, spinach, gravy, chocolate, egg, cheese, clay, pigment, oil, and combinations thereof.
6. The method of claim 1, wherein the enzyme is applied to the swatch in combination with a detergent ingredient.
7. The method of claim 1, further comprising agitating the swatch and enzyme during incubation.
8. A method of assaying for a preferred detergent composition comprising:
 - a) providing a swatch of material comprising a piece of material and a stain;
 - b) fixing the stain to the material;
 - c) applying a detergent composition to the swatch; and
 - d) incubating the swatch and detergent composition.
9. The method of claim 8, further comprising measuring the degree of removal of the stain from the material.

10. The method of claim 8, wherein the material is selected from the group consisting of a fabric, plastic, glass, or ceramic.
11. The method of claim 8, wherein the stain is selected from the group consisting of blood, milk, ink, grass, spinach, gravy, chocolate, egg, cheese, clay, pigment, oil, and combinations thereof.
12. The method of claim 8, wherein the detergent composition is applied to the swatch in combination with an enzyme.
13. The method of claim 12, wherein the enzyme is selected from the group consisting of a protease, a cellulase, an amylase, a laccase, and a lipase.
14. The method of claim 8, further comprising agitating the swatch and detergent composition during incubation.
15. A method of determining the catalytic efficiency of an enzyme comprising:
 - a) providing a swatch of material comprising a piece of material and a stain;
 - b) applying the enzyme to the swatch;
 - c) incubating the swatch and enzyme;
 - d) removing the swatch or supernatant; and
 - e) measuring a constituent of the stain.
16. The method of claim 15, wherein the enzyme is selected from the group consisting of a protease, a cellulase, an amylase, a laccase, and a lipase.
17. The method of claim 15, wherein the material is selected from the group consisting of a fabric, plastic or ceramic.
18. The method of claim 15, wherein the stain is selected from the group consisting of blood, milk, ink, grass, gravy, chocolate, egg, cheese, clay, pigment, oil, and combinations thereof.

19. The method of claim 15, wherein the enzyme is applied to the swatch in combination with a detergent ingredient.
20. The method of claim 15, further comprising agitating the swatch and enzyme during incubation.
21. The method of claim 15, wherein the constituent is ink from a BMI stain.
22. The method of claim 15, wherein the constituent is labeled blood from a BMI stain.
23. The method of claim 15, wherein the constituent is in the supernatant.
24. The method of claim 15, wherein the constituent is measured by absorbance of the constituent.
25. The method of claim 15, wherein the constituent is measured by the fluorescence of the constituent.

1 / 1

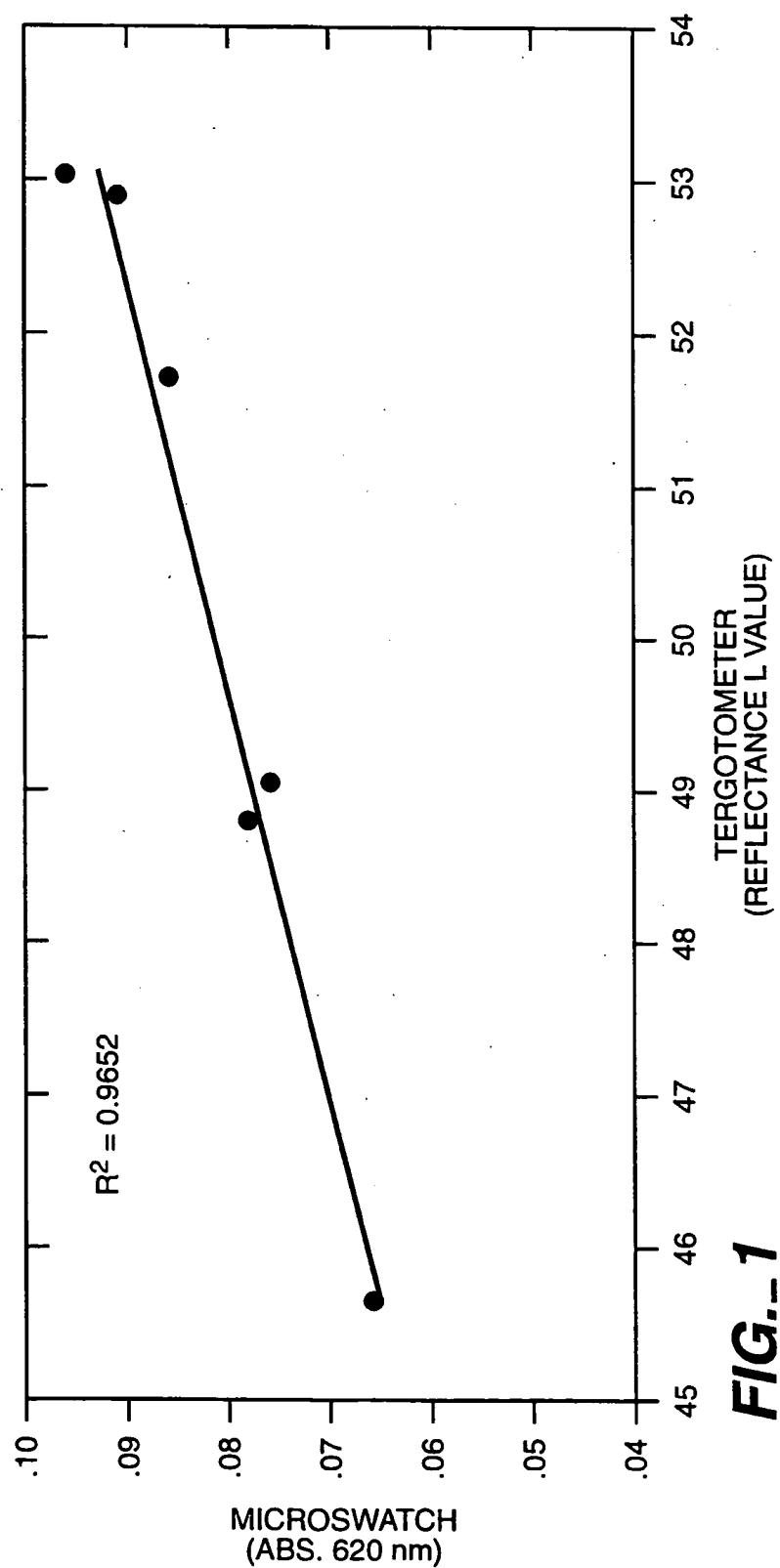


FIG. 1

THIS PAGE BLANK (USPTO)